Case reports

Chronic *Burkholderia cepacia* bronchiectasis in a non-cystic fibrosis individual

M J Ledson, M J Gallagher, M J Walshaw

Abstract

Infection with *Burkholderia cepacia* due to social contact is well described in patients with cystic fibrosis. However, social transmission to non-cystic fibrosis individuals or chronic colonisation in non-cystic fibrosis individuals has not been described. A report of *B cepacia* bronchiectasis is presented where a previously healthy mother of two cystic fibrosis children colonised with *B cepacia* became infected by the same epidemic strain. The implications of this for parents, siblings, and partners of individuals with cystic fibrosis are discussed.

(Thorax 1998;53:430–432)

Keywords: *Burkholderia cepacia*; cross infection

*Burkholderia cepacia* is a well recognised pathogen in patients with cystic fibrosis, immunocompromised patients, and those undergoing mechanical ventilation. Rare cases of acute non-pulmonary *B cepacia* infection have also been described in immunocompetent patients. Transmission is either nosocomial or, in the case of cystic fibrosis, by social contact. However, social transmission to or chronic colonisation in non-cystic fibrosis individuals has not been described. We present a case of chronic *B cepacia* bronchiectasis in the mother of two children with cystic fibrosis already colonised with *B cepacia*.

Case report

A 47 year old non-smoking woman with an unremarkable previous medical history presented to her GP with persistent right pleuritic chest pain in September 1995. A chest radiograph showed vague shadowing in the right upper zone and she was treated with analgesia and oral co-amoxiclav. A repeat chest radiograph showed little change and, although her symptoms remained, no immediate further action was taken. Three months later she was referred to her local district general hospital complaining of increasing malaise and more chest pain. A further chest radiograph showed progression of the right upper zone shadowing and a diagnosis of tuberculosis was considered.

She was not producing sputum and fiberoptic bronchoscopy was carried out in order to obtain microbiological samples. This revealed an inflamed right upper lobe orifice, washings from which grew a fully sensitive strain of *Haemophilus influenzae*. She had a two week course of co-amoxiclav with no benefit. Direct smear examination of the washings showed no evidence of tuberculosis.

One month later she presented to the local accident and emergency department complaining of progressive malaise, weight loss, and pyrexia and a further chest radiograph showed marked worsening of the right lung shadowing (fig 1). She was transferred to our unit because of the possibility that she was suffering from tuberculosis. On admission she was pyrexial (38.5°C), tachypnoeic, and mildly hypoxaemic (Pao2, 9.6 kPa). She had lost 6 kg in weight over the preceding two months. There were crackles over the right upper lobe. Her white cell count was 15 400 (82% neutrophils, rest of differential count normal). A Mantoux test was negative and she was unable to produce sputum. An HIV test was negative, serum immunoglobulins showed a non-specific polyclonal increase, IgG subclasses showed no isolated deficiencies, autoantibodies were negative, ANCA test was negative, and blood sugar and ACE levels were in the normal range. She was commenced speculatively on quadruple antituberculous chemotherapy and oral steroids and intravenous ceftazidime. However, she continued to deteriorate and a chest CT scan showed extensive consolidation in the right upper and middle lobes, now with peripheral consolidation in the left lung. She underwent rigid bronchoscopic examination and an open right lung biopsy specimen was taken. The surgeon who undertook this noted that the whole of the right lung was very inflamed, typical of an acute infective process. Histological examination of the open lung biopsy specimen merely revealed an acute inflammatory process. Washings taken at rigid bronchoscopy, however, grew only *Burkholderia cepacia* which was immediately sensitive to ceftazidime and co-trimoxazole but resistant to all other antibiotics tested. Subsequently, sputum culture grew *B cepacia*.

Anti-tuberculous chemotherapy was stopped and she was commenced on high dose intravenous ceftazidime and co-trimoxazole. Following this her pyrexia gradually settled and her appetite and weight increased. All microbiological samples sent for tuberculous culture were ultimately negative. After six weeks in hospital she was discharged home on a reducing course of steroids and oral co-trimoxazole. All subsequent sputum cultures have grown *B cepacia* and a further CT scan in July 1996 showed bronchiectasis in the right middle and upper lobes. She has since required one further
hospital admission for an exacerbation of *B cepacia* infection and this organism continues to be the only one in her limited daily sputum sample. Although she remains well, simple spirometric tests are now only 70% predicted.

This patient has had nine children, two of whom suffer from cystic fibrosis. She has been intimately involved in the care of these children, preparing and administering their nebulised antibiotics and bronchodilators and helping them with their physiotherapy. Both children became colonised by *B cepacia* in 1991. Microbiological screening of the rest of the family has failed to reveal any other members colonised by this organism. Genetic testing revealed both children to be DF508/621+1(G>T) and the mother to be heterozygous for DF508. Extensive DNA screening tests have failed to reveal a further cystic fibrosis gene for the mother, and her sweat chloride level is only 8 mmol/l (low normal range).

Pulse field gel electrophoresis of genomic cepacia DNA has been shown to give different patterns for organisms from different sources and this method has become accepted as the gold standard for epidemiological typing of *B cepacia*. It was therefore chosen to identify the relationship of the patient’s strain to those of her children with cystic fibrosis. The patterns derived from the three strains were identical, proving them to be from the same source (fig 2). Polymerase chain reaction (PCR) for the cable pilus also established that all three strains possessed the gene for the pilus and were therefore related to the “epidemic” strain (data not shown).

**Discussion**

In patients with cystic fibrosis the transmission of *B cepacia* depends on many factors. High numbers of *B cepacia* (>10⁸ cfu/ml) are present in the saliva of colonised patients and it has been shown that indirect spread via contaminated fomites is possible. Whilst airborne dissemination may present a small risk of acquisition, the highest risk occurs in the direct exchange of respiratory secretions associated with kissing and the intimate social contact which occurs between family members. Different *B cepacia* strains differ greatly in their rates of transmission. In the UK a very transmissible strain of *B cepacia* has been identified which is identical to a strain from Ontario, Canada. This strain, labelled ET 12 or UK “epidemic strain”, has a unique form of pilus designated “cable pilus” due to its length (2 µm) and intertwining properties. Up to 40% of patients in UK cystic fibrosis centres are colonised by *B cepacia*, 38% of which is due to the “epidemic strain”, involving 50% of cystic fibrosis centres. Transient colonisation can occur with some strains of *B cepacia*, but individuals who acquire the epidemic strain invariably remain chronically colonised.

In non-cystic fibrosis patients *B cepacia* pneumonia is characteristically a hospital acquired infection in the intubated or immunocompromised. There are rare case reports of community acquired *B cepacia* pneumonia occurring in previously healthy individuals but there are no reported cases of chronic respiratory colonisation.

This patient appears to have developed chronic respiratory colonisation with the epidemic strain of *B cepacia* following an acute infection with the organism acquired from one of her two affected offspring. Whilst it is still possible that she is a “forme fruste” of cystic fibrosis, we have been unable to detect a second gene despite extensive first and second level screening (ruling out 99% of cystic fibrosis genes) and she has a negative sweat test. Furthermore, she has had nine children and reached the age of 47 years without exhibiting any other symptoms. We are not aware of any other such cases, either where *B cepacia* has been transmitted from cystic fibrosis patients to immunocompetent adult individuals or where chronic colonisation and lung damage with the organism has been the end result.
This is a potentially worrying development for the parents, siblings, and partners of individuals with cystic fibrosis who are necessarily intimately exposed to the microbiological pathogens carried by cystic fibrosis patients.


Cross infection between cystic fibrosis patients colonised with Burkholderia cepacia

M J Ledson, M J Gallagher, J E Corkill, C A Hart, M J Walshaw

Abstract

Whilst patient to patient spread of the respiratory pathogen Burkholderia cepacia is well recognised between patients with cystic fibrosis, prompting a strict segregation policy, cross colonisation between cystic fibrosis patients already infected with B cepacia has not been described and surveys show a very low incidence of patients with more than one strain. Five adult cystic fibrosis patients with B cepacia are presented who became cross colonised with a second B cepacia (UK epidemic) strain, four of whom then died, three from the cepacia syndrome. These cases show that, amongst segregated patients, cross colonisation with different B cepacia strains is possible, and even in these patients the acquisition of the UK epidemic strain may have a fatal outcome. In future it may be necessary to segregate cystic fibrosis patients colonised with the UK epidemic strain from all other patients with cystic fibrosis.

Keywords: Burkholderia cepacia; cystic fibrosis; cross infection

Respiratory colonisation of patients with cystic fibrosis with Burkholderia cepacia can cause an accelerated fall in pulmonary function and 20% of cases develop fatal acute fulminant pneumonia (the cepacia syndrome). The spread of B cepacia between individuals with cystic fibrosis due to social contact is well recognised and, because of this, a strict segregation policy between B cepacia colonised (BC+) and non-colonised (BC–) patients is advocated in all cystic fibrosis units.

In the UK a very transmissible strain of B cepacia has been identified and labelled electrophoretic type 12 (ET 12) or “UK epidemic” strain, and is present in over 50% of clinics in the UK. Furthermore, patients who are already colonised with B cepacia are usually allowed to mix freely with each other, raising the possibility that cross colonisation with this strain may occur. Despite this, there have been no studies to determine whether cross colonisation with B cepacia occurs in this patient group, and surveys in Britain and Ireland have shown a very low incidence of patients colonised with more than one B cepacia strain.

A number of adult cystic fibrosis patients have been transferred to our clinic from the local paediatric centre who were already colonised with the UK epidemic B cepacia strain, and five other patients colonised with non-epidemic B cepacia have been transferred mainly from peripheral paediatric clinics. In keeping with the national policy, we segregated these BC+ patients from those who were BC–, but all BC+ patients were allowed to mix socially, attend the same outpatient clinic, and were admitted to the same wards for inpatient treatment. All five of the non-epidemic BC+ patients subsequently became cross colonised with the UK epidemic B cepacia strain. Furthermore, four of these rapidly succumbed, three from the “cepacia syndrome”. We present the methods used to type the B cepacia strains in our clinic and also the case histories of these five patients.

B cepacia typing method

The separate B cepacia strains were differentiated by pulsed field gel electrophoresis (PFGE)
Cross infection between CF patients colonised with *B cepacia*

POLYMERASE CHAIN REACTION (PCR) RIBOTYPING
Chromosomal template DNA was extracted by the Chelex-100 resin method. Ten colonies of organism were emulsified in 5% (w/v) Chelex solution, boiled for 10 minutes, vortexed for 30 seconds and then centrifuged at 10 000g for one minute. The supernate was pipetted into Eppendorf tubes and refrigerated for use in PCR. Oligonucleotide primers were designed to span conserved regions of the 16S and 23S regions of the rRNA operon. The sequences of primers used were 5'-TTGTACACACCGGCCGTTCA-3' for the 16S region and 5'-GGTGACCTTAGATGTTTCAGTTC-3' for the 23S region. Amplification was performed in a mixture of 10mM Tris-HCl (pH 8.8), 2.0 mM magnesium chloride, 0.1% W-1, 0.9 M Tris-HCl, 0.9 M boric acid, and 200 µM deoxynucleoside triphosphates, and 100 pmol each of primer. Approximately 20 ng of template DNA was used in each amplification. The total volume of the reaction was brought up to 50 µl with distilled H₂O and 200 µM deoxynucleoside triphosphates, and 100 pmol each of primer. Approximately 20 ng of template DNA was used in each amplification. The total volume of the reaction was brought up to 50 µl with distilled H₂O and 2.5 U of *Taq* DNA polymerase. An initial denaturing step of 95°C for three minutes was followed by 30 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, except for an extension step of four minutes during the last cycle. The products were then separated by electrophoresis for 2.5 hours at 100 V in 1.8% agarose. 100 bp lambda ladders (Bio-Rad) were used as DNA size standards.

PULSED FIELD GEL ELECTROPHORESIS (PFGE)
The bacterial cells were grown overnight in BHI broth, harvested by centrifugation, washed in cell suspension buffer (10 mM Tris, 20 mM NaCl, 50 mM EDTA) and mixed with equal volumes of pulsed field certified agarose (Bio-Rad) containing 4 µl lysozyme. The mixture was dispensed into 100 µl insert molds and allowed to solidify on ice. The plugs were incubated in 40 µl lysozyme buffer (10 mM Tris, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) for one hour at 37°C. After washing in wash buffer (20 mM Tris, 50 mM EDTA) the plugs were placed in proteinase K buffer (100 mM EDTA, 0.2% sodium deoxycholate, 1.0% sodium lauryl sarcosine) containing 1 mg of proteinase K and incubated overnight at 50°C. The plugs were then washed in wash buffer with 20 µl of 100 mM PMSF for 0.5 hour. After four further washes of 0.5 hour in wash buffer the plugs were cut with 2 µl Spe I (Bio-Rad) in 300 µl restriction enzyme buffer (20 mM Tris-HCl, 5 mM MgCl₂, 50 mM KCl) and incubated at 37°C for 18 hours. Restriction fragments were separated by PFGE using a CHEF DR II system (Bio-Rad) through 1% (w/v) pulsed field certified agarose in 0.5 × TBE (1 × TBE is 0.9 M Tris-HCl, 0.9 M boric acid, and 1.0 mM EDTA). Electrophoresis was performed for 20 hours at 0.5 × TBE buffer at 14°C with an initial and final pulse time of five and 35 seconds, respectively. Lambda concatamers were used as DNA size standards. The resulting gel pattern was analysed using a Bio-Rad fingerprinting programme to produce a dendrogram of genetic relatedness. The results were corroborated independently by the Epidemiological Typing Laboratory, Central Public Health Laboratory, London.

CABLE PILUS TYPING BY PCR
The method for amplification is identical to that for PCR ribotyping except primers spanning the cable pilus gene replace the 16S and 23S primers. The sequence of primers used was: sense primer 5'-CCAAAGGACTAACCAG-3' and antisense primer 5'-ACGCGGATGCATCCATACA-3'. It produces an amplicon of 676 bp.

Case histories

**CASE 1**
An 18 year old man who had cystic fibrosis (DF508/DF508) diagnosed at the age of 20 months had been colonised by *Pseudomonas aeruginosa* for many years. In 1992 he became colonised with a unique strain of *B cepacia*. Lung function initially fell and then stabilised and he was requiring four courses of intravenous antibiotics per year. He was admitted in July 1996 with a two week history of increased sputum production and a day history of increasing breathlessness. He was pyrexial (38°C), and had a neutrophil count of 23 000. There were widespread coarse crepitations on auscultation and the chest radiograph showed florid bilateral consolidation. He was commenced on high dose intravenous cotrimoxazole, colomycin, and piperacillin to which previous organisms cultured from sputum were sensitive. Sputum cultured on admission grew a second strain of *B cepacia* (UK epidemic strain). He deteriorated and died on the ninth day of admission from “cepaacia syndrome”.

**CASE 2**
A 34 year old man diagnosed with cystic fibrosis (DF508/DF508) at the age of four months had been colonised with *Pseudomonas aerugino- nosa* for many years. A unique strain of *B cepacia* was intermittently cultured from sputum between November 1992 and November 1993 and continually from then on. Lung function initially fell and then stabilised. By 1996 he was requiring four admissions per year for intravenous antibiotic therapy. He presented in September 1996 with a one week history of increasing sputum production and haemoptysis. On examination he was clubbed, afebrile, and auscultation of the chest revealed coarse crepitations in all zones. He had a mild neutrophilia (8800) and mild hypoaxaemia (PaO₂ 10.0 kPa on breathing air). His chest radiograph was unchanged from recent...
previous radiographs. He was started on high dose intravenous ceftazidime and tobramycin to which organisms previously cultured were sensitive. On the tenth day of admission his temperature spiked to 39°C, his sputum production increased, and he became tachypnoeic. His neutrophil count rose to 12,100 and he had worsening hypoxaemia (PaO2 7.1 kPa on breathing room air). The chest radiograph showed florid bilateral consolidation (fig 1) and sputum culture revealed a pure growth of *B cepacia* (UK epidemic strain).

The antibiotics were changed to intravenous colomycin, ceftazidime, co-trimoxazole, and oral chloramphenicol. Despite this, he rapidly succumbed to the “cepacia syndrome”.

**CASE 3**

A 21 year old man was diagnosed with cystic fibrosis (DF508/N1303K) at the age of 18 months. He had been growing *P aeruginosa* in his sputum for many years. In April 1994 he became colonised by *B cepacia* (unique strain). Lung function remained stable until July 1994, requiring five courses of intravenous antibiotics per year, when a second strain of *B cepacia* was isolated (UK epidemic strain) and the original *B cepacia* strain could no longer be cultured. Lung function deteriorated and he required continuous intravenous antibiotics until his death from respiratory failure in May 1997.

**CASE 4**

A 20 year old man was diagnosed with cystic fibrosis (DF508/R553X) at the age of eight months. He had been growing *P aeruginosa* in his sputum for many years. He was colonised by *B cepacia* (unique strain) before referral to our unit in 1994. In July 1994 he presented with worsening shortness of breath and increasing sputum production. He was pyrexial (39.1°C) with a neutrophilia (37,400) and hypoxaemia (PaO2 4.8 kPa on breathing room air). There were widespread crepitations and wheezes throughout his chest and the chest radiograph revealed extensive shadowing bilaterally with confluent shadowing at the right base. Sputum culture showed a second strain of *B cepacia* (UK epidemic strain). Despite treatment with intravenous co-trimoxazole, colomycin and ceftazidime he rapidly succumbed to the “cepacia syndrome”.

**CASE 5**

A 25 year old man was diagnosed with cystic fibrosis at the age of 10 months. He had been growing *P aeruginosa* in his sputum for many years. He was colonised by *B cepacia* (unique strain) in March 1994. Lung function initially fell and then stabilised at a low level (FEV1 30% predicted). In April 1997 a second strain of *B cepacia* was isolated (UK epidemic strain) and the original *B cepacia* strain could no longer be cultured. To date there has been no change in his clinical condition.

Both PFGE and PCR ribotyping distinguished each original isolate to be of a unique genotype (figs 2 and 3), whilst the subsequent dendrogram proved the secondary isolates to be indistinguishable from each other and to the prevalent strain in the clinic. Only these secondary isolates possessed the cable pilus gene (fig 4), as did those from all of the other patients in the clinic colonised with *B cepacia*, therefore confirming the strain as the transatlantic ET 12 (UK epidemic) clone.
Cross infection between CF patients colonised with B cepacia

Discussion

The “epidemic” strain of B cepacia was first isolated in the UK in August 1989 and by 1996 it had been isolated in 50% of UK cystic fibrosis centres and from 38% of all B cepacia samples submitted for genotypic analysis. The prevalence of B cepacia in some UK centres has now reached the 40% described in some North American studies. In our centre 37 of a total clinic population of 121 adult cystic fibrosis patients have been colonised by B cepacia, and 24 are still alive. Once colonised by B cepacia patients can have varying clinical outcomes. Whilst some remain asymptomatic, others have an accelerated decline in lung function and up to 20% succumb to an overwhelming fatal pneumonia and septicaemia, the “cepacia syndrome”. Recent studies from the USA have suggested that the increased risk associated with colonisation with B cepacia in patients with cystic fibrosis is three times that of uncolonised individuals, and that the average life expectancy is almost halved.

The transmission of B cepacia in patients with cystic fibrosis depends on many factors, and different strains of B cepacia vary greatly in their rate of transmissibility and transient colonisation can occur. Whilst in some cystic fibrosis patients the source of B cepacia colonisation is unclear, there is no doubt that patient to patient transmission of the epidemic strain can occur and individuals who acquire this strain invariably remain chronically colonised (authors’ unpublished data). This epidemic strain has a high rate of transmission: a patient harbouring two strains of B cepacia transmitted only the epidemic strain to his girlfriend. High numbers of B cepacia (>10^6 cfu/ml) are present in the saliva of colonised patients and indirect spread via contaminated fomites is possible. B cepacia has been grown from nebulisers used by colonised individuals and in a study looking at airborne dissemination up to 158 cfu/ml of organisms were recovered from the air of a room occupied by a colonised patient. Contamination of the environment by sputum has been shown to persevere for weeks and thus indirect transmission may occur from contaminated surfaces. However, the highest risk occurs in the direct exchange of respiratory secretions associated with kissing and the intimate social contact which occurs between family members. Because of these factors, most centres now advocate a strict segregation policy between B cepacia colonised and non-colonised cystic fibrosis patients, in an attempt to limit the spread of this organism throughout the cystic fibrosis population. However, there have been no attempts to separate patients colonised with different genotypes of B cepacia from each other. Indeed, in our clinic cepacia patients were free to socialise with each other whilst inpatients and many of them maintained social contact when in the community.

Colonisation with more than one strain of B cepacia is unusual, having been reported in less than 10% of patients, and there have been no reported cases of cross colonisation with different B cepacia strains in individuals who already carry one such genotype. This may be because, although other authors have noted phenotypic variations in B cepacia strains from the same patient, conventional ribotyping techniques are unable to separate them. In order to overcome this three molecular techniques were applied in our study to the B cepacia strains to establish their relatedness to each other and to the UK epidemic strain. Firstly, PCR amplification patterns of the intergeneric spacer regions between the 16S rRNA and 23S rRNA was used, a technique shown in 1992 to be capable of separating epidemiologically unrelated isolates of B cepacia and which has been successfully applied to B cepacia strains in other clinics. Secondly, PFGE of genomic DNA digested with a rare cutting restriction endonuclease was used. This method has been shown

Figure 3 Pulsed field gel electrophoresis in 1% pulse field agarose after cutting with Spe 1. Lane 1, 1 kb lambda DNA ladder; lane 2, original isolate from patient 1; lane 3, secondary isolate from patient 1; lane 4, original isolate from patient 2; lane 5, secondary isolate from patient 2; lane 6, original isolate from patient 3; lane 7, secondary isolate from patient 3; lane 8, original isolate from patient 4; lane 9, secondary isolate from patient 4; lane 10, original isolate from patient 5; lane 11, secondary isolate from patient 5.

Figure 4 Cable pili PCR products analysed by agarose gel electrophoresis. Lane 1, 1 kb lambda DNA ladder; lane 2, original isolate from patient 1; lane 3, secondary isolate from patient 1; lane 4, original isolate from patient 2; lane 5, secondary isolate from patient 2; lane 6, original isolate from patient 3; lane 7, secondary isolate from patient 3; lane 8, original isolate from patient 4; lane 9, secondary isolate from patient 4; lane 10, original isolate from patient 5; lane 11, secondary isolate from patient 5.
to give different patterns for organisms from different sources,20 is more specific than ribotyping, and has become the gold standard technique in molecular epidemiology.21 Thirdly, specific PCR for the unique pilus form22 of the UK epidemic strain, labelled “cable” pilus due to its length (2 µm) and propensity to intertwine, was used. In all the patients reported here PFGE and ribotyping established the initial B cepacia strains to be of unique genotypes. The strain subsequently acquired in each case was identical to the prevalent strain in the clinic, and PCR for the cable pilus gene established this strain to be of the UK epidemic genotype. Recently, two other epidemiology factors have been described which show a high correlation with the presence of cable pili.23 24

This study shows for the first time that, even amongst segregated patients, cross colonisation with epidemic B cepacia strains can occur. Indeed, all our B cepacia patients are now colonised by the epidemic strain. Furthermore, in four cases this was associated with a fatal outcome, suggesting that the acquisition of two strains of B cepacia and, particularly, the secondary acquisition of the epidemic strain has a worse prognosis than single strain colonisation alone. In future it may be necessary to segregate cystic fibrosis patients colonised with the UK epidemic strain from all other cystic fibrosis patients, including those with other strains of B cepacia. In our clinic we have now adopted a policy of isolating new patients with B cepacia from all other patients until the genotype of their strain has been established, and the strain genotypes of existing cepacia patients are regularly reviewed.

We thank Fiona Clode and Mary Kaufmann from the Hospital Infection Laboratory, Central Public Health Laboratory, Colindale, London. The work of M J Gallagher was supported by a grant from the Cardiothoracic Centre.

Cross infection between cystic fibrosis patients colonised with *Burkholderia cepacia*

M J Ledson, M J Gallagher, J E Corkill, C A Hart and M J Walshaw

*Thorax* 1998 53: 432-436
doi: 10.1136/thx.53.5.432

Updated information and services can be found at:
http://thorax.bmj.com/content/53/5/432

These include:

**References**
This article cites 22 articles, 10 of which you can access for free at:
http://thorax.bmj.com/content/53/5/432#BIBL

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections

- Cystic fibrosis (525)
- Drugs: infectious diseases (968)

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/